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(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yuichi Hikichi et al.

Application No.: 10/500,216

Confirmation No.: 3278

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Art Unit: 1652

For: PREVENTIVES/REMEDIES FOR CANCER

Examiner: Mohammady Meah

Commissioner for Patents
P.O. Box 1450
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DECLARATION UNDER 37 CFR 1.132

I, Yuichi HIKICHI, the undersigned, a citizen of Japan residing at 4-21-2-1-504, Matsushiro, Tsukuba-shi, Ibaraki 305-0035, JAPAN do hereby declare:

1. That I am an employee of the Assignee of the above-identified application, That I graduated from Kyoto University with degree of Master of Science in March 1988,

That I have been employed by Takeda Pharmaceutical Company, Ltd., Osaka, Japan, since April, 1988, and have been engaged in pharmaceutical research of said company,

That I am one of the inventors of the above-identified patent application,

That the following experiments were carried out by myself or under my direction:

2. Discovery of SUV39H1 inhibitor.

2.1 Materials and methods

Recombinant enzymes.

Recombinant human SUV39H1 protein was produced and purified as described in the present application. As for RPMT1, recombinant protein was purchased from Upstate.

Enzyme assay.

Twenty μl of assay buffer consisting of 125 mM Tris/HCl (pH 8.5), 12.5 mM KCl, 25 mM $MgCl_2$, 12.5% Glycerol, 6.25 mM DTT, 2.5 μM $ZnCl_2$, and 5 μl of 20 ng/μl Histone H3 or H4 peptide (Upstate) solution and 10 μl of enzyme solution obtained above were mixed in the streptavidin-coated well (ScintiPlate Streptavidin Covalent, Wallac). Then, 5 μl of DMSO (negative control) or Chetomin was added to the assay mixture, followed by addition of 10 μl of ^{3}H-S-Adenosyl Methionine (^{3}H-SAM, final 0.5 μCi). Incubations were carried out for 3 hr at 37C. Radioactivity was measured using liquid scintillation counter in accordance with manufacturer's manual (Wallac).

2.2 Results and discussion

As a result of inhibitor screening, Chetomin (Figure 1) was identified as a SUV39H1 inhibitor.

Indeed, Figure 2A shows that Chetomin inhibited SUV39H1 effectively in a dose dependent manner. In order to confirm the selectivity, recombinant RPMT1, which is a protein arginine methyltransferase and is known to methylate Histone H4-Arg3[1)], was assayed with ^{3}H-SAM and Histone H4 N-terminal peptide. However, Chetomin could not inhibit the methyltransferase activity of RPMT1 (Figure 2B).

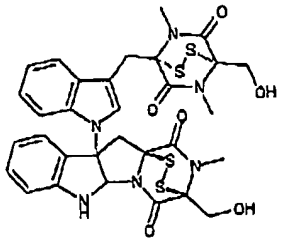

Figure 1 Chemical structure of Chetomin

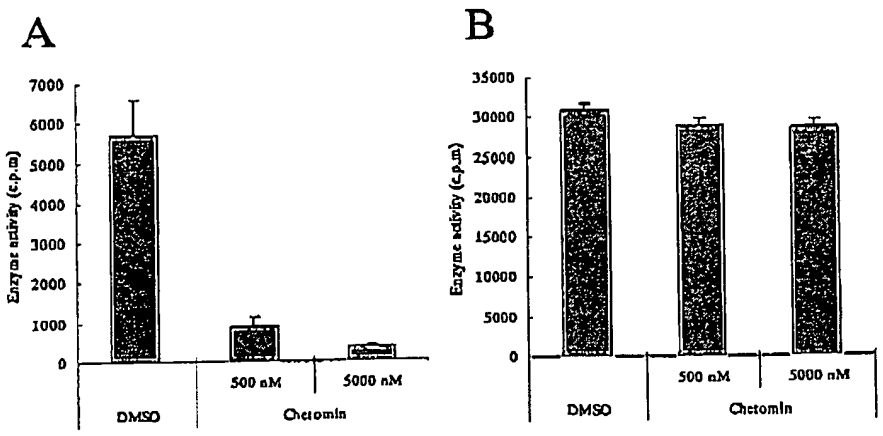

Figure 2, Enzyme Inhibition by Chetomin. Enzyme assays were performed using SUV39H1 (A) or RPMT1 (B). Radioactivity, which is derived from the transferred methyl residue, was counted 3 hrs after the addition of ^{3}H-labeled SAM into the reaction mixture.

Recently, Greiner et al. reported that Chaetocin (Figure 3), which had the similar structure to Chetomin, inhibited not only Drosophila SU(VAR)3-9 but also human SUV39H1[2]. In addition, they showed that Chaetocin was more potent against these enzymes than the other methyltransferases, such as E(z)complex and G9a.

These results suggest that Chetomin and its derivatives could be the specific SUV39H1 inhibitor.

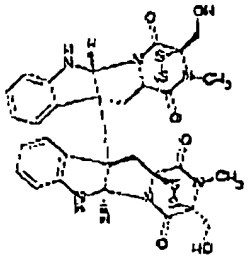

Figure 3, Chemical structure of Chaetocin

3. Inhibition of Cell Growth

3.1 Materials and methods

Cell Culture.

MDA-MB-231 (human breast cancer cell line) was purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's MEM (Invitrogen) supplemented with 10% fetal bovine serum.

Growth inhibition and apoptosis induction.

MDA-MB-231 cells were seeded at 3×10^3 cells per well in 96-well plate (Falcon). After 24 hrs' cultivation, Chetomin was added to the cells at the concentration ranging from 10^{-10} M to 10^{-7} M. In order to analyze cell proliferation, WST-8 kit (Dojindo, Japan), which contains a water-soluble tetrazolium salt, was used. Cell Death Detection ELISA (Roche), which can detect fragmented DNA, was used for analyzing apoptosis. Both proliferation and apoptosis detection assay were performed 72hrs after the addition of Chetomin in accordance with the manufacturer's protocol.

3.2 Results and discussion

As it was mentioned in the present application, MDA-MB-231 cell line has been selected for the study of antisense oligonucleotides. Figure 5A showed that Chetomin also inhibited the cell growth of MDA-MB-231. In addition, it induced apoptosis (Figure 5B). Although the methods for the assays were different, the level of anti- tumor activity in vitro was almost the same with that of NCI database[3], in which the growth inhibition activity was measured by SRB method.

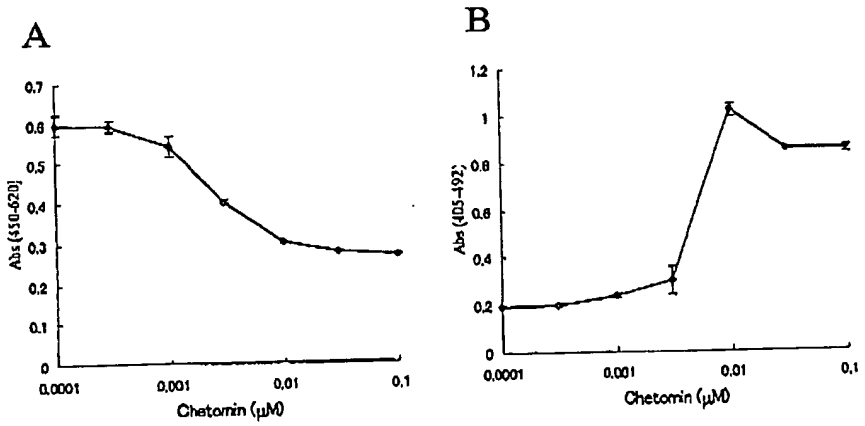

Figure 5, Chetomin inhibited growth and induced apoptosis to MDA-MB-231. A breast cancer cell line, MDA-MB-231, was cultured for 72 hrs with Chetomin. The growth was measured using WST8 kit (A) and the induction of apoptosis was measured using Cell Death Detection ELISA (B)

Recently, Kung et al. reported that Chetomin was identified as a disrupter of HIF binding to p300[4]. They also demonstrated that Chetomin showed the significant anti-tumor activity in PC3 xenograft model.

References

1) Strahl BD, Briggs SD, Brame CJ, Caldwell JA, Koh SS, Ma H, Cook RG, Shabanowitz J, Hunt DF, Stallcup MR and Allis CD. (2001) *Curr. Biol.* 11, 996-1000

2) Greiner D, Bonaldi T, Eskeland R, Roemer E and Imhof A. (2005) *Nat. Chem. Biol.* 1, 143-145.

3) http://dtp.nci.nih.gov/docs/dtp_search.html

4) Kung AL, Zabludoff SD, France DS, Freedman SJ, Tanner EA, Vieira A, Cornell-Kennon S, Lee J, Wang B, Wang J, Memmert K, Naegeli HU, Petersen F, Eck MJ, Bair KW, Wood AW and Livingston DM. (2004) *Cancer Cell.* 6, 33-43

4. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing therein.

Date: Oct. 25, 2006

Yuichi Hikichi

Yuichi Hikichi